Coordinated regulation of transcription factors through *Notch2* is an important mediator of mast cell fate

Mamiko Sakata-Yanagimoto*†, Etsuko Nakagami-Yamaguchi*, Toshiki Saito*†, Keiki Kumano*†, Koji Yasutomo‡, Seishi Ogawa*§, Mineo Kurokawa†, and Shigeru Chiba*¶||

*Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; †Department of Hematology Oncology, and §21st Century Center Of Excellence Program, Graduate School of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; ‡Department of Immunology and Parasitology, Institute of Health Biosciences, University of Tokushima Graduate School, Kuramoto-cho, Tokushima, Tokushima 770-8503, Japan; and ¶Department of Hematology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba, Ibaraki 305-8575, Japan

Communicated by Francis H. Ruddle, Yale University, New Haven, CT, March 20, 2008 (received for review October 12, 2007)

Mast cells are thought to participate in a wide variety of pathophysiological conditions. Mechanisms of regulation, however, of mast cell production and maturation are still to be elucidated. Mast cell developmental process is likely to be profoundly affected by cell-autonomous transcriptional regulators such as the GATA family and CCAAT/enhancer binding protein (C/EBP) family members. Extracellular regulators such as stem cell factor and IL-3 have essential roles in basal and inducible mast cell generation, respectively. The relationship, however, between the extracellular signaling and cellular transcriptional control is unclear, and the trigger of the mast cell development remains elusive. Notch signaling plays a fundamental role in the lymphopoietic compartment, but its role in myeloid differentiation is less clear. Here, we demonstrate that Notch signaling connects environmental cues and transcriptional control for mast cell fate decision. Delta1, an established Notch ligand, instructs bone marrow common myeloid progenitors and granulocyte-macrophage progenitors toward mast cell lineage at the expense of other granulocyte-macrophage lineages, depending on the function of the *Notch2* gene. Notch2 signaling results in the up-regulation of Hes-1 and GATA3, whereas simultaneous overexpression of these transcription factors remarkably biases the progenitor fate toward the mast cell-containing colony-forming cells. C/EBPα mRNA was down-regulated in myeloid progenitors as a consequence of Hes-1 overexpression, in agreement with the recent proposal that the down-regulation of C/EBPα is necessary for mast cell fate determination. Taken together, signaling through Notch2 determines the fate of myeloid progenitors toward mast cell-producing progenitors, via coordinately up-regulating Hes-1 and GATA3.

CCAAT/enhancer binding protein α | GATA3 | Hes-1 | Delta1 | myeloid progenitor

Mast cells are thought to participate in a wide variety of physiologic and pathologic processes. In addition to their involvement in allergic disorders and protective immune responses to parasites, mast cells are important in a broader sense in both innate and acquired immunity (1, 2). Mast cells migrate from the bone marrow at an immature differentiation stage and complete the maturation process in the peripheral tissues under the influence of local growth and differentiation factors (2). These environmental cues tailor the mast cell phenotype to carry out functions specific to each peripheral tissue.

The main factors influencing mast cell number and phenotype include stem cell factor (SCF), which is a ligand for c-Kit, IL-3, and T helper type II (Th2) -associated cytokines, such as IL-4 and IL-9 (2). Signal transduction through the phosphatidylinositol 3-kinase pathway, Ras-mitogen-activated protein kinase pathway, Janus kinase-signal transducer and activators of transcription pathway (3), etc., in the presence of IL-3 and SCF, might coordinate the lineage-specific transcription factors, but there is a disconnect between our understanding of cytokine signaling and transcription factor regulation. The generation of mast cells has been shown to depend on cooperative interplay between regulatory proteins: PU.1 up-regulation, GATA2 up-regulation (4), and CCAAT/enhancer binding protein α (C/EBPα) down-regulation (5).

Notch receptor-mediated signaling has a fundamental role in cell fate determination in a variety of animals. In the mammalian immune system, Notch signaling is involved in the commitment and differentiation of T cells, development of splenic marginal zone B cells, and differentiation and functional modulation of mature T cells (6, 7). Questions remain, however, about whether and how Notch signaling regulates immune cells other than T and B cells. To the best of our knowledge, there are only two reports (8, 9) describing the relationship between Notch signaling and mast cells. According to those papers, Jagged-1, a Notch ligand (8), and Notch2 (9), are highly expressed in mast cells.

Here, we demonstrate that Notch signaling has a significant role in mast cell development. Using *Notch2*–null bone marrow cells, we show that Notch2 signaling promotes cell fate determination of bone marrow progenitors toward the progenitors having mast cell differentiation capacity at the expense of those that lose it. Furthermore, we demonstrate that the coordinated regulation of two transcription factors, Hes-1 and GATA3, determines mast cell fate under Notch signaling. Overexpression of Hes-1 in myeloid progenitors represses C/EBPα mRNA expression, in agreement with the proposal that the C/EBPα down-regulation provides essential condition for mast cell development (5).

These findings suggest that Notch signaling, a key regulator of lymphocytes, more broadly affects cells of the immune system through dynamic regulation of transcription factors.

Results

Notch2-Mediated Signaling Facilitates Mast Cell Lineage Development at the Expense of Granulocyte/Macrophage Development from Both Common Myeloid Progenitors (CMPs) and Granulocyte-Macrophage Progenitors (GMPs) *in Vitro*. We cultured isolated CMPs and GMPs in SCF, IL-3, IL-6, and thrombopoietin (TPO) for 7 days with plate-fixed Delta1-Fc chimeric protein, a soluble Notch ligand.

Author contributions: M.S.-Y., and S.C. designed research; M.S.-Y. and E.N.-Y. performed research; T.S. contributed new reagents/analytic tools; M.S.-Y. and S.C. analyzed data; and M.S.-Y., K.K., K.Y., S.O., M.K., and S.C. wrote the paper.

The authors declare no conflict of interest.

||To whom correspondence should be addressed. E-mail: schiba-tky@umin.ac.jp

This article contains supporting information online at www.pnas.org/cgi/content/full/0801074105/DCSupplemental.

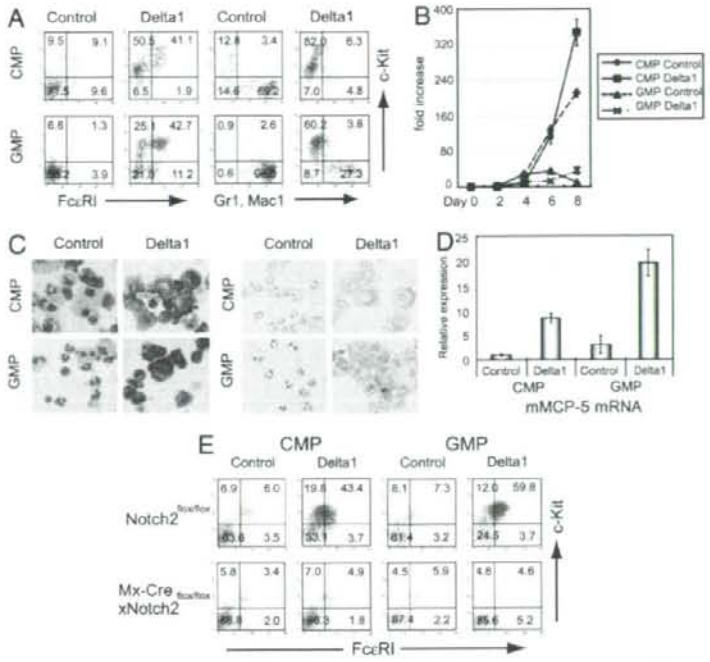

Fig. 1. Notch2-mediated signaling facilitates mast cell lineage development at the expense of granulocyte/macrophage development from CMPs and GMPs *in vitro*. (*A*) Sorted CMPs or GMPs of WT mice were cultured with plate-fixed Delta1-Fc chimeric protein or control Fc protein for 7 days in 50 ng/ml SCF, 20 ng/ml IL-3, 20 ng/ml IL-6, and 20 ng/ml TPO. FACS analysis showed FcεRI$^+$c-Kit$^+$Gr1$^-$Mac1$^-$ mast cells were highly enriched with Delta1-Fc, whereas Gr1$^+$Mac1$^+$ granulocytes and macophages were the main population with control Fc protein. (*B*) Cell growth in culture of CMPs and GMPs with Delta1-Fc or control Fc protein. (*C*) Day-7 progeny of CMPs and GMPs were analyzed morphologically by cytospin. (*Left*) Wright-Giemsa staining (original magnification: ×400). (*Right*) Toluidine blue staining (original magnification: ×400). (*D*) mRNA was extracted from day-7 progeny of CMPs and GMPs, reverse-transcribed, and applied to quantitative real-time PCR analysis for mouse mast cell protease-5 (mMCP-5). Data are presented as mean +/− SD; $n = 4$, $P = 0.0286$ (both CMPs and GMPs, 7 days) (nonparametric test). (*E*) CMPs and GMPs of Mx-Cre x *Notch2*$^{flox/flox}$ and littermate *Notch2*$^{flox/flox}$ mice were cultured with Delta1-Fc or control Fc protein for 7 days in SCF, IL-3, IL-6, and TPO. *Notch2*-null CMPs and GMPs failed to respond to Delta1-Fc, whereas the results obtained with littermate mice treated with pIpC were virtually the same as those obtained with WT mice.

In the culture using control Fc protein, instead of Delta1-Fc, the vast majority of the cells were Gr1$^+$Mac1$^+$ granulocytes/macrophages. Compared with this, Lin$^-$c-Kit$^+$FcεRI$^+$ mast cells were highly enriched, whereas there were fewer Gr1$^+$ or Mac1$^+$ granulocytes/macrophages with Delta1-Fc (Fig. 1*A*). The cell number was not significantly different in the culture with Delta1-Fc from the culture with the control Fc protein on day 7, when the flowcytometric analysis was performed (Fig. 1*B*). As shown, time courses of cell growth were different between the cultures with Delta1-Fc and control, because of the difference in the differentiation fate. Morphological observation confirmed the conversion from granulocytes/macrophages with the control Fc to mast cells, which contained toluidine blue-positive granules, with Delta1-Fc (Fig. 1*C*). The expression level of mast cell-specific protease, mast cell protease-5, was higher in Delta1-stimulted population compared with control (Fig. 1*D*).

Whereas all of Notch1, Notch2, and Notch3 are expressed in myeloid progenitors [supporting information (SI) Fig. S1], Notch2 is known to be expressed in mast cells among Notch 1–4 receptors (9). We sorted CMPs and GMPs from poly(deoxyinosinic/deoxycytidylic) acid (pIpC)-treated Mx-Cre x *Notch2*$^{flox/flox}$ (N2-MxcKO) mice (10), and then stimulated them with Delta1-Fc. The mast cell increase by Delta1-Fc was strikingly blunted with *Notch2*-null CMPs or GMPs, whereas the results obtained with littermate *Notch2*$^{flox/flox}$ mice treated with pIpC were virtually the same as those obtained with WT mice (Fig. 1*E*; compare with Fig. 1*A*). These observations indicate that Notch2-mediated signaling plays a key role in derivation of mast cells from myeloid progenitor cells.

Hes-1 Is Up-Regulated Downstream of Notch Signaling but Is Insufficient for Mast Cell Derivation. Quantitative real-time PCR analysis revealed that Delta1-Fc treatment of CMPs or GMPs up-regulated mRNA for Hes-1, a basic helix–loop–helix transcriptional repressor that often functions downstream of Notch signaling (11), within as little as 8 h (Fig. 2*A*). To investigate whether Hes-1 is a downstream effector of Notch signaling for mast cell enrichment by Delta1-Fc, we retrovirally expressed Hes-1 in CMPs and GMPs. Expression of the Hes-1 protein was confirmed by a Western blot analysis of the retrovirally infected NIH 3T3 cells (Fig. 2*B*). Derivation of the Lin$^-$c-Kit$^+$FcεRI$^+$ mast cells, however, was not increased by Hes-1 expression after 8 d of culture (Fig. 2*C*), whereas there was a relative increase in Lin$^-$c-Kit$^+$FcεRI$^-$ cells. This observation indicated that the effect of Hes-1 expression on mast cell enrichment was not equivalent to that of Delta1-Fc stimulation, suggesting the presence of an effector molecule other than Hes-1 at the downstream of Notch.

GATA3, but Not GATA2, Is a Mediator Downstream of Notch2 for Mast Cell Developmental Decision, Together with Hes-1. Among the GATA family of transcription factors, GATA2, which is up-

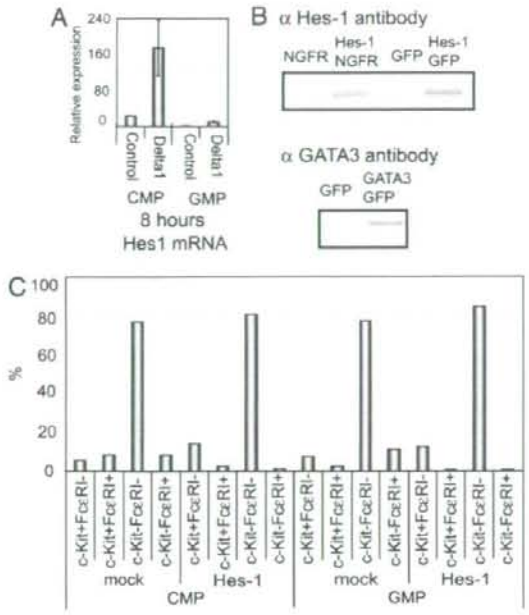

Fig. 2. Hes-1 up-regulation downstream of Notch signaling is insufficient for mast cell derivation. (*A*) CMPs and GMPs were stimulated with plate-fixed Delta1-Fc protein or control Fc protein in SCF, IL-3, IL-6, and TPO. Cells were collected at 8 h, and Hes-1 mRNA was measured by quantitative real-time PCR analysis. Hes-1 mRNA was substantially up-regulated with Delta1-Fc compared with control. Data are presented as mean +/− SD; $n = 4$, $P = 0.0286$ (both CMPs and GMPs, 8 h) (nonparametric test). (*B*) A retrovirus packaging cell line, PLAT-E, was transfected with cDNA-subcloned retrovirus vectors, i.e., Hes-1-GCDNsam/IRES-NGFR, Hes-1-pMYs/IRES-GFP, and GATA3-pMYs/IRES-GFP, or a mock retrovirus vector. NIH 3T3 cells were infected with the conditioned medium by using polybrene. The proteins were detected by anti-Hes-1 antibody (H-140; Santa Cruz) and anti-GATA3 antibody (HG3–31). There is a faint band migrating in the NGRF and GFP lanes. This may represent endogenously expressed Hes-1. (*C*) CMPs and GMPs were retrovirally transduced with Hes-1-pMYs/IRES-GFP or mock virus. The proportion of c-Kit$^+$FcεRI$^+$ mast cells was not significantly increased by Hes-1 expression. The proportion of c-Kit$^+$FcεRI$^-$ cells, which probably represent myeloid progenitors, was always greater in the Hes-1-expressing population than control.

regulated during Notch-mediated inhibition of granulocytic differentiation (12, 13), is proposed to be a regulator of mast cell differentiation (4, 5, 14). Nevertheless, GATA2 expression levels in CMPs or GMPs stimulated with Delta1-Fc were equal to or lower than those stimulated with control Fc protein during the first 48 h (Fig. 3*A*).

GATA3 is a crucial transcription factor during T cell development. Recently, it was reported that its overexpression in double negative (DN) 1 and DN2 stage thymocytes blocked further T cell development, and unexpectedly, induced mast cells from these early thymocyte populations (15). Physiologic relevance between GATA3 and mast cell generation, however, has yet to be elucidated. Delta1-Fc stimulation of CMPs and GMPs substantially increased GATA3 mRNA during a relatively later time course (Fig. 3*B*). This finding suggests that GATA3 is a possible target of Notch signaling, albeit indirect, for mast cell production.

Thus, we examined whether Hes-1 and GATA3 are bona fide effectors functioning downstream of Notch signaling for mast cell production, by retrovirally transducing CMPs and GMPs with these genes (Fig. 3 *C* and *D*). Both CMP- and GMP-derived fractions expressing Hes-1 alone [with the nerve growth factor receptor (NGFR) marker] gave rise to populations very similar to those derived from the fraction expressing Hes-1 with the GFP marker (Figs. 2*C* and 3 *C* and *D*). There was no consistent increase in the c-Kit$^+$FcεRI$^+$ mast cell population at day 8, although it appears that the Hes-1 single-positive CMP-derived fraction gave rise to this population slightly more than control in Fig. 3 *C* and *D*. On the contrary, the Hes-1 single-positive fractions gave rise to fewer mast cells than the control virus-infected fractions at the later time course (data not shown). In the fractions expressing GATA3 alone (with the GFP marker), the c-Kit$^+$FcεRI$^+$ mast cell population only marginally, although reproducibly, increased compared with controls (Fig. 3 *C* and *D*).

In contrast to these findings, the c-Kit$^+$FcεRI$^+$ mast cell population was markedly enriched in the Hes-1 and GATA3 double-positive fractions at day 8 after the infection of CMPs and GMPs compared with either mock or Hes-1 or GATA3 single-positive fractions (Fig. 3 *C* and *D*).

We reproducibly observed increases in the c-Kit$^+$FcεRI$^-$ population, which might represent the expanded progenitors maintaining the immature state, in the Hes-1 single-positive fractions. The c-Kit$^-$FcεRI$^+$ population, whose identification is unknown, variably increased in the GATA3 single-positive fractions compared with controls (Fig. 3*C*).

When plated in methylcellulose after the retroviral infection, Hes-1- and GATA3-coexpressing CMPs and GMPs formed large and monotonous cell-containing colonies (plating efficiency, ≈20% in CMPs and <10% in GMPs); staining indicated that >80% of colonies contained mast cells, positive for toluidine blue, as the major population (Fig. 3 *E–G*), whereas the plating efficiency was comparable to that with mock introduction (Fig. 3*E*).

These observations indicate that the simultaneous expression of GATA3 and Hes-1 biased the cell fate of myeloid progenitors toward the downstream progenitors having mast cell-generating capacity at the single-cell level, rather than that those transcription factors expanded the pool of mast cell progenitors or mast cells.

Hes-1 Up-Regulation Causes C/EBPα Down-Regulation. C/EBPα is a critical transcription factor for myeloid differentiation (16), and its down-regulation cooperates with the up-regulation of a GATA transcription factor to instruct mast cell development (5). At 48 h after the initiation of Hes-1 retroviral transduction, C/EBPα mRNA was down-regulated in both CMPs and GMPs (Fig. 4*A*). The C/EBPα mRNA level was also markedly suppressed in a mouse myeloid cell line 32D that stably expressed exogenous Hes-1 (Fig. 4*B*). These findings suggest that the C/EBPα down-regulation is the major outcome of Hes-1 up-regulation induced by Delta1–Notch2 signaling in the myeloid progenitors.

Discussion

In the present study, we demonstrate that Notch2-mediated signaling has a significant role in the mast cell derivation from myeloid progenitors such as CMPs and GMPs. This biological effect is probably mediated through coordinated up-regulation of Hes-1 and GATA3 through Notch2 signaling; Hes-1 up-regulation further results in C/EBPα down-regulation, which is important for the blockade of myeloid lineage differentiation. It is not unexpected that Hes-1 up-regulation plays a role in the execution of Notch2 signaling, given the fact that Hes-1 is an established target of Notch signaling in a number of cell systems (17). However, we show in this article that Hes-1 up-regulation represents only a part of signaling downstream of Notch activation. Exogenous Hes-1 expression in CMPs and GMPs resulted in the increase of Lin$^-$c-Kit$^+$FcεRI$^-$ cells, obviously different

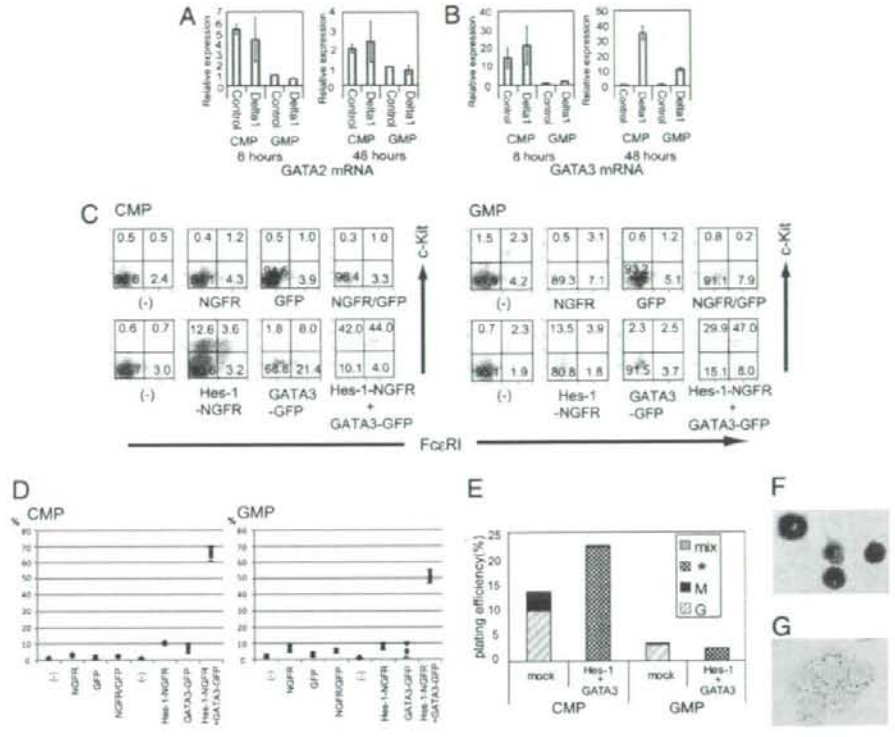

Fig. 3. GATA3, but not GATA2, is a mediator downstream of Notch2 for mast cell developmental decision, together with Hes-1. (*A*) CMPs and GMPs were stimulated with plate-fixed Delta1-Fc in the presence of SCF, IL-3, IL-6, and TPO for 8 or 48 h. Cells were harvested, and quantitative real-time PCR was performed. GATA2 mRNA levels were slightly decreased rather than increased by Delta1-Fc in GMPs at 8 h, and not significantly different in CMPs treated with Delta1-Fc and control Fc at 8 h or in CMPs and GMPs treated with Delta1-Fc and control Fc at 48 h. (*B*) Quantitative real-time PCR analyses in CMPs and GMPs revealed that GATA3 mRNA was up-regulated by Delta1-Fc at 48 h, but not at 8 h. Data are presented as mean +/− SD; $n = 4$, $P = 0.6857$ (CMPs, 8 h); $n = 4$, $P = 0.0571$ (GMPs, 8 h); $n = 4$, $P = 0.0286$ (both CMPs and GMPs, 48 h) (nonparametric test). (*C*) CMPs and GMPs were retrovirally transduced with Hes-1-GCDNsam/IRES-NGFR and GATA3-pMYs/IRES-GFP and subjected to FACS analysis 8 days after the infection. The c-Kit$^+$FcεRI$^+$ fraction was remarkably enriched in Hes-1 and GATA3 double-positive fraction compared with the Hes-1 or GATA3 single-positive fraction or the mock virus-introduced fraction. (*D*) The proportions of c-Kit$^+$FcεRI$^+$ fraction are depicted. Each diamond represents a data point. The bars represent +/− 2 SD. CMP, $n = 6$; GMP, $n = 4$. (*E*) NGFR and GFP double-positive cells were sorted at 48 h after infection. Two hundred and fifty NGFR and GFP double-positive CMPs and 1,500 GMPs were plated per dish and cultured for 7 days in methylcellulose, supplemented with SCF, IL-3, IL-6, and TPO. Hes-1- and GATA3-coexpressing cells formed mainly mast cell colonies, whereas mock virus-transduced cells formed various colonies including granulocyte, macrophage, or a mixture of these cells. G, granulocyte colonies; M, macrophage colonies; mix, granulocyte/macrophage or mixed colonies; •, colonies mainly consisting of mast cells. The result of a representative experiment is shown ($n = 2$). (*F*) Wright-Giemsa staining of Hes-1 and GATA3 coexpressing colony-forming cells. (Original magnification: ×400.) (*G*) Toluidine blue staining of Hes-1 and GATA3 coexpressing colony-forming cells. (Original magnification: ×400.)

from the Lin$^-$c-Kit$^+$FcεRI$^+$ mast cell enrichment that was seen by the Delta1 stimulation. Although the identity of the Lin$^-$c-Kit$^+$FcεRI$^-$ cells has yet to be determined, these cells may contain immature myeloid progenitors that could differentiate into mast cells if GATA3 would coexist, while granulocytes/macrophages would if other critical molecules (such as C/EBPα) would coexist.

We identified GATA3, not GATA2, up-regulation that complements Hes-1 up-regulation and creates a part of Notch signaling. Involvement of GATA2 is proposed to be important for inhibiting granulocytic differentiation downstream of Notch signaling in both the 32D cell line (13) and mouse hematopoietic progenitor cells (12). Furthermore, GATA2 is required for *in vitro* mast cell generation (4, 14), and enforced expression of GATA2 instructs the C/EBPα-deficient myeloid progenitors and common lymphoid progenitors to become mast cells *in vitro* (5). Our conclusion might appear to be inconsistent with those of other articles. We confirmed that the enforced GATA2 and Hes-1 coexpression in CMPs and GMPs resulted in predominant mast cell generation in a manner indistinguishable from that of GATA3 and Hes-1 coexpression (data not shown). This finding indicates that GATA2 and GATA3 have redundant properties when they are expressed exogenously. The result of colony formation assay from CMPs and GMPs with enforced GATA3 and Hes-1 expression indicates that the mast cell derivation is based on the cell fate alteration made in the individual progenitor cells. Because the biological readout for Notch ligand stimulation was virtually the same as that for the GATA3 and Hes-1 coexpression, the substantial mast cell generation at day 7 with Delta1-Fc is likely to be caused mainly by biased cell fate decision in the myeloid progenitors.

In a different line, the introduction of GATA3 alone to thymocytes was recently reported to result in mast cell generation (15). Although this report might appear to be inconsistent

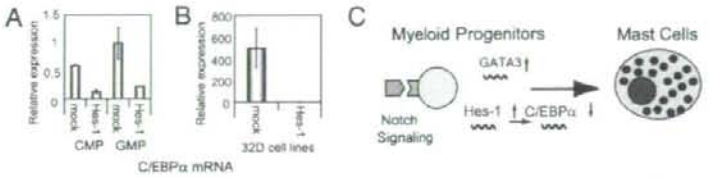

Fig. 4. Hes-1 up-regulation causes C/EBPα down-regulation. (*A*) CMPs and GMPs were retrovirally transduced with Hes-1-pMYs/IRES-GFP. GFP$^+$ cells were sorted 48 h after infection and examined for C/EBPα mRNA by quantitative real-time PCR. C/EBPα mRNA in CMPs and GMPs was substantially reduced by Hes-1 compared with mock virus transduction. Data are presented as mean +/− SD; $n = 5$ (mock) or 4 (Hes-1), $P = 0.0159$ (CMP); $n = 5$, $P = 0.0079$ (GMP) (nonparametric test). (*B*) 32D cells stably transduced with Hes-1 and maintained in 5 ng/ml IL-3 were examined for C/EBPα expression. C/EBPα mRNA was reduced in Hes-1-transduced clones compared with mock-transduced clones. Data were confirmed by experiments using two independent clones. Data are presented as mean +/− SD. (*C*) Schematic model of Notch signaling in the mast cell system.

with our data, the difference in the starting cell populations could cause the different results. We conclude that the Hes-1 expression, and probably subsequent C/EBPα down-regulation, is required, although not sufficient, for mast cell generation from CMPs and GMPs. In contrast, C/EBPα is already down-regulated in thymocytes at the DN1 and DN2 stages (18), and thus, introduction of GATA3 might be sufficient for mast cell generation from early thymocytes. As for the relationship between GATA3 and Notch signaling, both Notch1 and Notch2 are important for the generation of Th2 cells and act by directly inducing transcription of GATA3 and IL-4 (19, 20). It is, however, unclear whether such a direct regulation is applicable to cells in other lineages. In our observation, GATA3 was up-regulated by Delta1-Fc at 48 h but not at 8 h in CMPs and GMPs, making it obscure whether GATA3 is a direct target of Notch signaling in these cells. The role of IL-4 in the regulation of GATA3 in these cells remains to be determined.

We and others previously reported that enforced expression of Hes-1 in a 32D cell line inhibits granulocytic differentiation induced by granulocyte colony-stimulating factor (13, 21). In the present study, we demonstrated that C/EBPα down-regulation occurs downstream of Hes-1 in both the 32D cell line and fresh CMPs and GMPs. C/EBPα repression by Hes-1 was previously shown to be among the mechanisms of Notch-Hes-1-mediated inhibition of adipogenesis from a preadipocyte cell line (22). Although less remarkable compared with Hes-1 overexpression, Delta1-Fc stimulation also induced C/EBPα repression, in a time course after Hes-1 up-regulation. This finding suggests that the physiologic Hes-1 up-regulation is sufficient for C/EBPα repression (data not shown). Our data, thus, support a paradigm that the Notch-Hes-1-C/EBPα axis consists of a common pathway for differentiation inhibition in a variety of cell lineages.

It was recently suggested that down-regulation of C/EBPα followed by up-regulation of a GATA factor orchestrates mast cell differentiation from myeloid progenitors (5). This could be true, but importantly, we demonstrated that such a balanced regulation of transcription factors is a result of environmental signaling through Notch2, rather than a cell-autonomous operation (Fig. 4*C*).

The physiological significance of Notch2-mediated cell fate bias toward mast cell lineage remains to be determined, because mast cells were not depleted in naïve status in N2-MxcKO mice (M.S.-Y. and S.C., unpublished data). Cultured mast cells were also generated from Notch2-null bone marrow cells as efficiently as WT bone marrow cells. Notably, mast cells are not depleted in mice lacking the IL-3 gene, whereas IL-3 is the most potent mast cell developmental factor *in vitro*. However, IL-3-deficient mice are defective in mast cell-mediated intestinal nematode eradication. The pathways and mechanisms responsible for regulating mast cell progenitor recruitment and trafficking are likely to be dynamic and susceptible to modification during inflammation (1). Similarly, Notch2 is required for the proper response of mast cells during nematode infection (M.S.-Y. and S.C., unpublished observation). Notch2-mediated mast cell derivation might also be required for such pathological settings, whereas it is unnecessary for the steady-state mast cell generation.

Materials and Methods

Mice. *Notch2*$^{flox/flox}$ mice have been described (10). Mx-Cre transgenic mice (23) were crossed with *Notch2*$^{flox/flox}$ mice, and the progeny were injected with pIpC (Sigma-Aldrich) seven times every other day from 3 d after birth (25 μg/g body weight) or three times between 4 and 6 wk of age (20 μg/g body weight). All experiments were done in accordance with institutional guidelines.

Myeloid Progenitors. Bone marrow cells from each mouse strain studied were incubated with biotinylated antibodies for lineage markers including anti-CD3, anti-CD4, anti-CD8, anti-B220, anti-Ter119, and anti-Gr-1 antibodies (BD Pharmingen) followed by incubation with streptavidin Micro Beads (Miltenyi Biotec). The lineage marker-negative fraction was separated with an autoMACS separator (Miltenyi Biotec) and incubated with anti-CD34-FITC, anti-CD16/32 (FcγIII/II receptor)-phycoerythrin (PE), anti-c-Kit-allophycocyanin (APC), streptavidin peridinin chlorophyll protein PerCP (BD Pharmingen), and anti-Scal-PE/Cy7 (eBioscience). Lin$^-$c-Kit$^+$Scal$^-$FcγR^{lo}CD34$^+$ and Lin-c-Kit$^+$Scal$^-$FcγR^{hi}CD34$^+$ cells (CMPs and GMPs, respectively) (24) were sorted by a FACSAria cell sorter (Becton Dickinson).

Ligand Fixation. Delta1-Fc has been described (25). A 24-well nontissue culture plate (Nalge Nunc) was coated with 10 μg/ml of rabbit anti-human IgG (DAKO), blocked with 20% FBS containing RPMI medium 1640 (Sigma-Aldrich), and washed with PBS. The Delta1-Fc (3.5 μg/ml) or Fc portion of human IgG (2 μg/ml, Fc protein; ART or Jackson ImmunoResearch Laboratories) was then incubated for 30 min, and supernatants were removed.

Ligand Stimulation of Myeloid Progenitors. Sorted CMPs or GMPs were cultured in Delta1-Fc or control Fc protein-fixed plates in 20% FBS containing Iscove's modified Dulbecco's medium (Sigma-Aldrich), supplemented with 50 ng/ml SCF, 20 ng/ml IL-3 (Peprotech), 20 ng/ml IL-6, and 20 ng/ml TPO (gifts from Kirin Pharma, Tokyo). On day 7, the cells were incubated with purified isotype IgE (BD Pharmingen) after blocking the Fcγ receptor with anti-CD16/32 (FcγIII/II receptor) antibody, stained with anti-IgE-FITC, anti-Gr-1-PE, anti-Mac1-PE, and anti-c-Kit-APC (BD Pharmingen), and then analyzed by FACSCalibur (Becton Dickinson). Cells cultured for 7 days were also characterized by Wright-Giemsa staining or toluidine blue staining (pH 0.5) on cytospin slides. In some experiments, mRNA was prepared at the indicated time points and quantified by real-time PCR as described below.

Retroviral Transduction. Hes-1 cDNA, a gift from R. Kageyama (Kyoto University, Kyoto, Japan), was subcloned into a retrovirus vector, GCDNsamIinternal ribosome entry site (IRES)-NGFR, a gift from H. Nakauchi (University of Tokyo) and M. Onodera (National Center for Child Health and Development, Tokyo). cDNAs for GATA3, a gift from S. Takahashi (University of Tsukuba), and Hes-1 were subcloned into pMYs/IRES-GFP, a gift from T. Kitamura (University of Tokyo). A retrovirus packaging cell line, PLAT-E (26), was transfected with each retrovirus vector by using FuGENE 6 (Roche Diagnostics). The conditioned medium was concentrated, placed in a 24-well nontissue culture dish for 4 h, and precoated with 40 μg/ml of RetroNectin (Takara Bio) overnight at 4°C. CMPs or GMPs were then plated for infection in the presence of 20% FBS, 50 ng/ml SCF, 20 ng/ml IL-3, 20 ng/ml IL-6, and 20 ng/ml TPO. Green fluorescent

proteins and/or human NGFR-positive fractions were subjected to FACS analysis 8 days after infection. Otherwise, the infected cells were sorted at 48 h from the initiation of infection with a FACSAria (Becton-Dickinson) cell sorter and used for colony assay using Methocult M3231 (StemCell Technologies), supplemented with cytokines as described above. Hes-1 stably expressing 32D cell lines were as described (13). To confirm protein expression, NIH 3T3 cells were also infected with the same viral supernatants by using polybrene (Sigma-Aldrich).

RNA Quantitation. Total cellular RNA was extracted with RNeasy (Qiagen) and converted to cDNA with SuperScript III (Invitrogen). GATA2, GATA3, C/EBPα, and mouse mast cell protease-5 were analyzed with *Taq*Man Gene Expression assays (Applied Biosystems). Hes-1 mRNA was measured as described (10). Real-time PCR was performed by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All of the data were standardized with 18s ribosomal RNA.

Western Blot Analysis. Virus-infected NIH 3T3 cells were solubilized in lysis buffer containing 1% Triton X-100. The Hes-1 and GATA3 proteins were detected by anti-Hes-1 antibody (H-140; Santa Cruz) and anti-GATA3 antibody (HG3–31), respectively.

Statistical Analysis. Results from two or three independent experiments (n = 2) of quantitative real-time PCR were analyzed by the Mann–Whitney test.

ACKNOWLEDGMENTS. We thank Dr. R. Kageyama for the Hes-1 cDNA; Dr. S. Takahashi for the GATA3 cDNA; Drs. H. Nakauchi and M. Onodera for the GCDNsam/IRES-NGFR; Dr. T. Kitamura for the pMYs/IRES-GFP and PLAT-E; Kirin Pharma for the TPO and IL-6; and Y. Mori for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research Grants 18013012, 19390258 (to S.C.), and 19890051 (to M.S.-Y.) from the Japan Society for the Promotion of Science/Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from the Sagawa Foundation for Promotion of Cancer Research (to S.C.).

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nature immunology

Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity

Yoichi Maekawa[1,8], Yoshiaki Minato[1,2,8], Chieko Ishifune[1], Takeshi Kurihara[1], Akiko Kitamura[1], Hidefumi Kojima[3], Hideo Yagita[4], Mamiko Sakata-Yanagimoto[5,6], Toshiki Saito[5], Ichiro Taniuchi[7], Shigeru Chiba[5,6], Saburo Sone[2] & Koji Yasutomo[1]

The acquisition of cytotoxic effector function by CD8⁺ T cells is crucial for the control of intracellular infection and tumor invasion. However, it remains unclear which signaling pathways are required for the differentiation of CD8⁺ cytotoxic T lymphocytes. We show here that Notch2-deficient T cells had impaired differentiation into cytotoxic T lymphocytes. In addition, dendritic cells with lower expression of the Notch ligand Delta-like 1 induced the differentiation of cytotoxic T lymphocytes less efficiently. We found that the intracellular domain of Notch2 interacted with a phosphorylated form of the transcription factor CREB1, and together these proteins bound the transcriptional coactivator p300 to form a complex on the promoter of the gene encoding granzyme B. Our results suggest that the highly regulated, dynamic control of T cell cytotoxicity depends on the integration of Notch2 and CREB1 signals.

The adaptive immune response is essential for purging a diverse repertoire of invading pathogens and transformed cells[1,2]. $CD8^+$ and $CD4^+$ T cells are required for successful eradication of intracellular pathogens or transformed cells. $CD8^+$ T cells recognize peptides displayed by major histocompatibility complex (MHC) class I molecules (peptide-MHC complexes) on target cells[3]. $CD8^+$ T cells use cytolytic and noncytolytic mechanisms to lyse target cells expressing foreign peptide-MHC complexes. These mechanisms involve perforin and granzymes, as well as cytokines such as interferon-γ (IFN-γ; A001238) and tumor necrosis factor[2–4].

$CD8^+$ T cells responding to specific peptide-MHC complexes undergo considerable changes in gene expression that regulate their differentiation into cytotoxic T lymphocytes (CTLs) and memory cells. Transcriptional regulators activated by T cell receptor (TCR) signaling induce the expression of genes important for directing proliferation, the acquisition of effector functions, and survival. Many transcription factors (such as NF-κB, AP-1 and Jun) expressed in a broad range of cell types are involved in the activation of $CD8^+$ T cells[3]. The T-box transcription factors T-bet and eomesodermin (Eomes) are important inducers of genes involved in the acquisition of $CD8^+$ T cell effector function and in the responsiveness to cytokines that regulate the survival of long-lived memory T cells[5,6]. However, it remains unclear how the transcription of genes encoding cytotoxic effector molecules in CTLs is regulated and how this process influences the direction and differentiation of effector or memory cells.

Notch signaling regulates cell fate 'choice' for a variety of cells[7–9]. Mammals express four Notch receptors and five Notch ligands. The Notch ligands are categorized into two families, Delta and Jagged[8–10], and mice and humans have three genes encoding Delta-like ligands (DL1, DL13 and DL14) and two genes encoding Jagged molecules (Jagged1 and Jagged2). Studies have shown that Notch signaling is involved in early T cell development as well as differentiation into mature $CD4^+$ T cells[7,9,11–19]. However, less is known about the function of Notch in mature $CD8^+$ T cells. One study has shown that constitutive overexpression of DL1 on alloantigen-bearing cells renders these cells nonimmunogenic and able to induce specific nonresponsiveness to a subsequent challenge with the same alloantigen[20]. That study also showed that ligation of Notch on splenic $CD8^+$ cells by purified DL1 protein results in much lower IFN-γ production and concomitantly more production of interleukin 10 (IL-10); these findings suggest that Notch signaling can alter the differentiation potential of $CD8^+$ T cells. However, it did not address whether physiological interaction of Notch with Notch ligands dictates the differentiation of CTLs or memory $CD8^+$ T cells. Furthermore, another report has shown that inhibition of Notch activation results in lower $CD8^+$ T cell proliferation and IFN-γ production[21]. However, that study analyzed Notch function through the use of a γ-secretase

[1]1Department of Immunology & Parasitology and [2]Department of Respiratory Medicine & Rheumatology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan. [3]Department of Immunology, Dokkyo Medical University, Tochigi 321-0293, Japan. [4]Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan. [5]5Department of Cell Therapy and Transplantation Medicine, University of Tokyo, Tokyo 113-8655, Japan. [6]Department of Clinical and Experimental Hematology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8575, Japan. [7]Laboratory for Transcriptional Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan. [8]These authors contributed equally to this work. Correspondence should be addressed to K.Y. (yasutomo@basic.med.tokushima-u.ac.jp).

Received 28 May; accepted 29 July; published online 24 August 2008; doi:10.1038/ni.1649

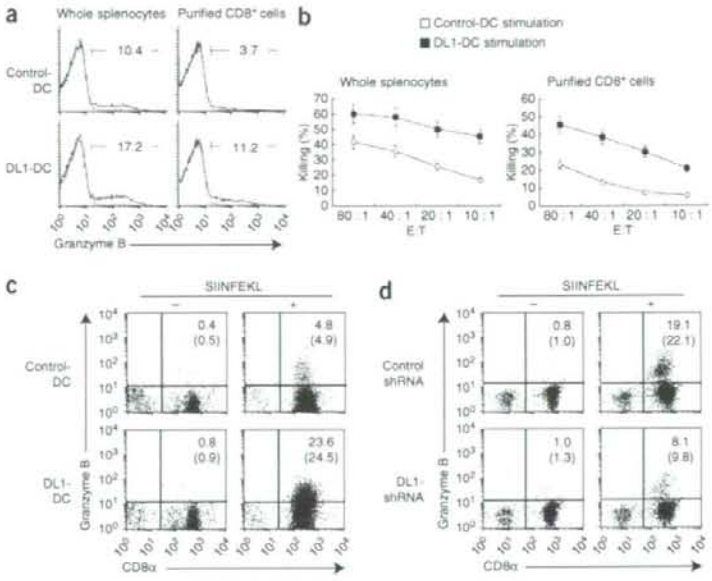

Figure 1 Notch signals control cytotoxic T cell differentiation *in vitro* and *in vivo*. (**a**,**b**) Flow cytometry (**a**) and ^{51}Cr-release assays (**b**) of total spleen T cells (Whole splenocytes) and purified CD8$^+$ cells from BALB/c mice, stimulated with DL1-DCs or control-DCs at a ratio of 10:1. (**a**) or at various ratios (**b**). (**a**) Granzyme B expression in CD8$^+$ T cells 3 d after stimulation. Numbers above bracketed lines indicate percent granzyme B–positive cells. (**b**) Cytolytic activity against EL-4 cells after 5 d of stimulation. E:T, effector/target. Data are representative of five independent experiments (error bars (**b**), s.d.). (**c**,**d**) Flow cytometry of granzyme B expression in splenocytes from B6 mice that received CD8$^+$ T cells from OT-I TCR–transgenic mice, then were inoculated twice with unpulsed (–) or OT-I peptide (SIINFEKL)-pulsed (+) control-DCs or DL1-DCs (**c**) or DCs transduced with DL1-specific shRNA (DL1-shRNA) or control shRNA (**d**), assessed 4 d after the second inoculation of DCs and gated on $V_\alpha2^+V_\beta5^+$ cells. Numbers in plots indicate percent granzyme B–positive cells (top) and percent total $V_\alpha2^+V_\beta5^+CD8^+$ cells among splenocytes (below in parentheses). Data are representative of at least four independent experiments.

inhibitor, which may affect other substrates instead of Notch specifically[8]. Therefore, the functions of Notch in CD8$^+$ T cell differentiation remain controversial.

In this report, we show that signaling mediated by Notch2 (A001671) was required for full cytotoxic activity of CTLs and that Notch2 directly controlled transcription of the gene encoding granzyme B (*Gzmb*), which is independent of Eomes. Furthermore, the transcriptional control of *Gzmb* by Notch2 signaling required the molecular interaction of Notch and the transcription factor CREB1 (A000690) in a stable complex on the *Gzmb* promoter. Our results indicate that the Notch and cyclic AMP–responsive element (CRE) pathways intersect to dynamically regulate transcription of CTL effector molecules. In addition, our findings suggest that Notch2 may act as a mechanism directing CD8$^+$ T cell cytolytic function in response to appropriate extrinsic stimuli.

RESULTS

DL1 promotes CTL differentiation

To assess the involvement of Notch signaling in CTL function, we stimulated total spleen cells or purified CD8$^+$ T cells from BALB/c mice with allogeneic bone marrow–derived dendritic cells (BMDCs) obtained from C57BL/6 (B6) mice and infected with a control retrovirus (control-DCs) or the same retrovirus encoding DL1 (DL1-DCs). The control-DCs expressed DL1, and infection with the DL1-encoding retrovirus further increased DL1 expression on the cell surface (**Supplementary Fig. 1a** online). Granzyme B production was much higher in total spleen cells and purified CD8$^+$ T cells after stimulation with DL1-DCs than after stimulation with control-DCs (**Fig. 1a**). Consistent with their higher expression of granzyme B, CD8$^+$ T cells stimulated with DL1-DCs lysed allogeneic EL-4 (mouse thymoma) target cells more efficiently than did CD8$^+$ T cells stimulated with control-DCs (**Fig. 1b**). The granzyme B production in CD8$^+$ T cells and the cytotoxic activity of CD8$^+$ T cells were higher for stimulated total spleen cells than for stimulated purified CD8$^+$ T cells. There was similarly less granzyme B production in splenocyte samples depleted of CD4$^+$ T cells than in total spleen cells (**Supplementary Fig. 1b**). These data suggest CD4$^+$ T cells stimulated by allogeneic spleen cells enhance the generation of CTLs. Published papers have shown that CD4$^+$ T cells are directly or indirectly involved in promoting the generation of CTLs[22,23]. We found that CD4$^+$ T cells cultured with allogeneic DCs promoted upregulation of DL1 expression on these DCs (**Supplementary Fig. 1c**); this observation suggests that CD4$^+$ T cells may be indirectly involved in providing Notch signals to CD8$^+$ T cells. Another possibility is that CD4$^+$ T cells activated by allogeneic DCs directly 'help' CTL differentiation, which is further enhanced by DL1 expressed on DCs. Although further analyses are needed to clarify the mechanism by which CD4$^+$ T cells enhance CTL differentiation in our experimental systems, these data indicate that DL1-mediated signals 'preferentially' direct CTL differentiation *in vitro*.

To test the hypothesis that DL1-mediated signaling contributes to CTL differentiation *in vivo*, we pulsed control-DCs or DL1-DCs with OT-I peptide (SIINFEKL) and injected the cells intravenously twice into B6 mice that had previously received T cells from mice transgenic for an OT-I T cell receptor (TCR), which express a $V_\alpha2V_\beta5$ TCR specific for SIINFEKL presented by H-2K^b. We evaluated the generation of OT-I-specific CTLs by staining the cells with $V_\alpha2$- and $V_\beta5$-specific antibodies 4 d after the second injection of peptide-pulsed DCs. The proportion of granzyme B–producing $V_\alpha2^+V_\beta5^+CD8^+$ T cells (called 'OT-I T cells' here) was higher in mice that received DL1-DCs than in those that received control-DCs (**Fig. 1c**), which indicates that DL1 can augment CTL differentiation *in vivo*.

We next investigated whether lower expression of DL1 on DCs impairs the generation of CTLs. We induced BMDCs *in vitro* with granulocyte-macrophage colony-stimulating factor and IL-4, retrovirally transduced the cells with control short hairpin RNA (shRNA) or DL1-specific shRNA, then pulsed them with OT-I peptide. The bicistronic retrovirus vector also encoded green fluorescence protein (GFP), which allowed us to identify infected cells and confirm shRNA-mediated suppression of DL1 surface expression (**Supplementary**

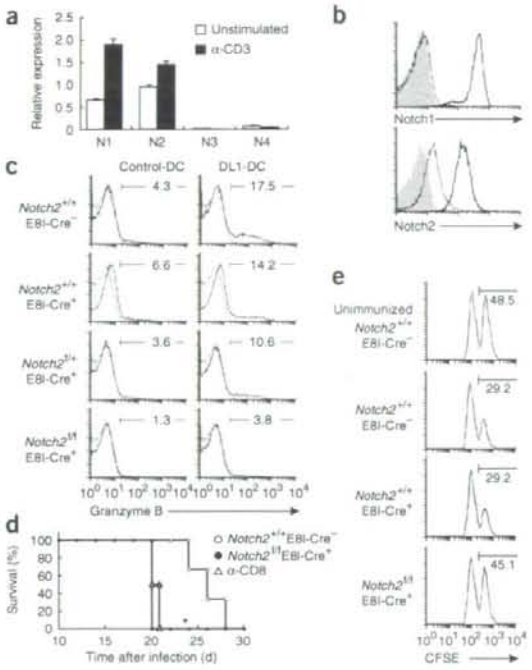

Figure 2 Notch2 on CD8⁺ T cells promotes CTL induction *in vitro* and *in vivo*. (**a**) Quantitative PCR of the expression of Notch1–Notch4 (N1–N4) in naive CD8⁺ T cells (Unstimulated) and activated CD8⁺ T cells stimulated with anti-CD3 (α-CD3), normalized to *Gapdh* mRNA and presented as 'fold increase' relative to *Notch2* expression in naive CD8⁺ T cells. Data are representative of three independent experiments (mean and s.d. of three samples). (**b**) Flow cytometry of the expression of Notch1 and Notch2 in total spleen cells (CD8⁺ T cells) left unstimulated (thin lines) or stimulated for 24 h with soluble anti-CD3 (5 μg/ml; thick lines). Shaded histograms, naive CD8⁺ cells stained with streptavidin-allophycocyanin alone. Data are representative of five independent experiments. (**c**) Flow cytometry of the expression of granzyme B in purified CD8⁺ cells from *Notch2*$^{+/+}$E8I-Cre⁻, *Notch2*$^{+/+}$E8I-Cre⁺, *Notch2*$^{f/+}$E8I-Cre⁺ or *Notch2*$^{f/f}$E8I-Cre⁺ mice, stimulated with BALB/c control-DCs or DL1-DCs, analyzed 3 d after stimulation. Thin lines, isotype-matched control antibody; thick lines, anti–granzyme B. Numbers above bracketed lines indicate percent granzyme B–positive cells among total CD8⁺ cells. Data are representative of five independent experiments. (**d**) Survival of mice after intraperitoneal infection with *T. cruzi* (2×10^3 trypomastigotes), assessed in *Notch2*$^{+/+}$E8I-Cre⁻ and *Notch2*$^{f/f}$E8I-Cre⁺ mice treated with control rat IgG and wild-type mice treated with antibody to mouse CD8β (α-CD8). *, $P = 0.0253$, wild-type versus *Notch2*$^{f/f}$E8I-Cre⁺ (log-rank test). Data are representative of three independent experiments. (**e**) Flow cytometry of CFSE expression in splenocytes from unimmunized *Notch2*$^{+/+}$E8I-Cre⁻ mice (top) or mice preimmunized with SIINFEKL-pulsed DCs (below), assessed 1 d after injection of a 1:1 mixture of SIINFEKL-pulsed spleen cells labeled with a high concentration of CFSE and unpulsed spleen cells labeled with a low concentration of CFSE. Numbers above bracketed lines indicate percent CFSE^{hi} cells. Data are representative of three independent experiments.

Fig. 1d). We isolated GFP⁺CD11c⁺ DCs and injected them intravenously into B6 mice previously injected with OT-1 T cells. Approximately 20% of splenic OT-1 T cells expressed granzyme B in mice that received DCs transduced with control shRNA, whereas only 8% did so in mice that received DCs transduced with DL1-specific shRNA (**Fig. 1d**). Furthermore, the absolute number of OT-I T cells in the spleen was also lower in mice that received DCs transduced with DL1-specific shRNA than in mice that received DCs transduced with control shRNA ($5.2 \times 10^5 \pm 1.2 \times 10^5$ cells per spleen versus $9.0 \times 10^5 \pm 1.3 \times 10^5$ cells per spleen, respectively; $n = 5$ mice per experiment; $P < 0.01$). These data collectively demonstrate that DL1 on DCs is required for optimal CTL differentiation *in vivo*, although it is possible that other cells expressing DL1 may contribute to CTL differentiation.

Notch2 controls CTL differentiation

Naive CD8⁺ T cells expressed Notch1 and Notch2 mRNA, and both transcripts were upregulated after stimulation with antibody to CD3 (anti-CD3; **Fig. 2a**). Naive and activated CD8⁺ T cells had extremely low expression of Notch3 and Notch4 (**Fig. 2a**). We detected expression of Notch2 but not of Notch1 on the surface of naive CD8⁺ T cells by flow cytometry (**Fig. 2b**). However, cell surface expression of both Notch1 and Notch2 was also rapidly upregulated after CD3 cross-linking (**Fig. 2b**).

To evaluate the contribution of Notch signaling to CTL differentiation, we crossed mice expressing a *lox*P-flanked (floxed) gene encoding Notch2 (*Notch2*$^{f/f}$) with mice expressing Cre recombinase driven by a combination of the core E8I enhancer and the *Cd8a* promoter (called 'E8I-Cre' here). E8I-Cre was expressed specifically in peripheral CD8⁺ T cells (data not shown). *Notch2*$^{f/f}$E8I-Cre⁺ mice lacked Notch2 expression in peripheral CD8⁺ T cells but not CD4⁺ T cells (**Supplementary Fig. 2a** online). CD8⁺ T cells from *Notch2*$^{f/f}$E8I-Cre⁺ mice had less production of granzyme B than did those from *Notch2*$^{+/+}$E8I-Cre⁻, *Notch2*$^{+/+}$E8I-Cre⁺ or *Notch2*$^{f/+}$E8I-Cre⁺ mice after stimulation with allogeneic control-DCs or DL1-DCs *in vitro* (**Fig. 2c**). CD8⁺ T cells from *Notch2*$^{f/+}$E8I-Cre⁺mice also had less production of granzyme B than did those from *Notch2*$^{+/+}$E8I-Cre⁻ or *Notch2*$^{+/+}$E8I-Cre⁺ mice, which indicated haploinsufficiency of Notch2 in terms of CTL differentiation.

To evaluate the effect of Notch2 deficiency on CTL induction *in vivo*, we infected *Notch2*$^{+/+}$E8I-Cre⁻ and *Notch2*$^{f/f}$E8I-Cre⁺ mice with the intracellular protozoan parasite *Trypanosoma cruzi* (**Fig. 2d**). The acute phase of *T. cruzi* infection is controlled by CTLs expressing granzyme B and perforin[24]. We monitored the survival of *Notch2*$^{+/+}$E8I-Cre⁻ and *Notch2*$^{f/f}$E8I-Cre⁺ mice after *T. cruzi* infection and found significantly earlier death of *Notch2*$^{f/f}$E8I-Cre⁺ mice than of *Notch2*$^{+/+}$E8I-Cre⁻ mice (**Fig. 2d**). Although the control mice eventually died after this infection, the mortality of *Notch2*$^{f/f}$E8I-Cre⁺ mice after *T. cruzi* infection was similar to that of wild-type mice depleted of CD8⁺ T cells (**Fig. 2d**), which indicates that Notch2 is a chief contributor to CD8⁺ T cell effector function in this experimental system. The expression of Cre itself in CD8⁺ T cells did not have any influence on the course of *T. cruzi* infection (**Supplementary Fig. 2b**). As the course of *T. cruzi* infection is affected by many factors other than CTLs, we used an *in vivo* CTL assay to further examine the importance of Notch2 in controlling CTL function (**Fig. 2e**). We immunized *Notch2*$^{+/+}$E8I-Cre⁻, *Notch2*$^{+/+}$E8I-Cre⁺ or *Notch2*$^{f/f}$E8I-Cre⁺ mice with OT-I peptide–pulsed DCs, then, 4 d later, gave the mice spleen cells labeled with the cytosolic dye CFSE (carboxyfluorescein diacetate succinimidyl diester) at a high concentration and pulsed with OT-I peptide or gave them spleen cells labeled with CFSE alone at a low concentration. We then compared the ratio of CFSE^{hi} cells to CFSE^{lo} cells at 24 h after cell transfer. *Notch2*$^{+/+}$E8I-Cre⁻ and *Notch2*$^{+/+}$E8I-Cre⁺ mice showed 'preferential' killing of peptide-pulsed spleen cells, whereas *Notch2*$^{f/f}$E8I-Cre⁺ mice showed little killing of either injected population (**Fig. 2e**). These data collectively indicate that Notch2 transduces an important signal for

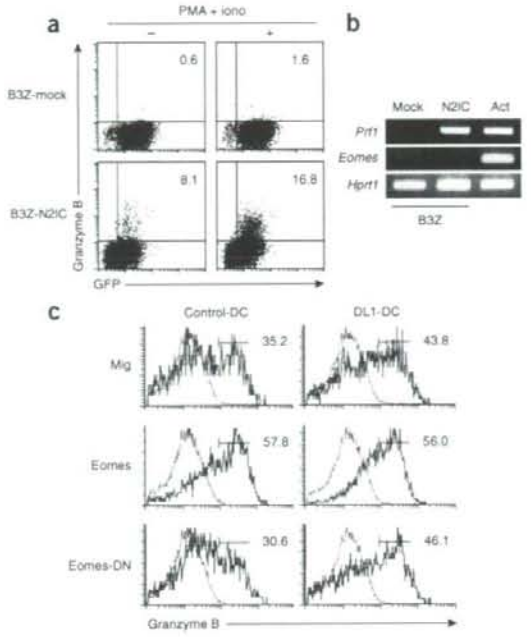

Figure 3 Notch2 signaling induces the transcription of genes encoding CTL effector molecules independently of Eomes. (**a**) Granzyme B expression in B3Z-N2IC and B3Z-mock cells with (+) or without (–) stimulation with PMA and ionomycin (PMA + iono). GFP encoded by the bicistronic retrovirus 'marks' infected cells; numbers in top right quadrants indicate percent granzyme B–positive GFP$^+$ cells. Data are representative of five independent experiments. (**b**) RT-PCR analysis of the expression of Eomes or perforin (*Prf1*) by B2Z-mock cells (Mock) or B3Z-N2IC cells (N2IC) or OT-I-transgenic CD8$^+$ T cells stimulated for 24 h with SIINFEKL peptide (Act). Data are representative of five independent experiments. (**c**) Flow cytometry of granzyme B expression by CD8$^+$GFP$^+$ cells among OT-I-transgenic CD8$^+$ T cells stimulated with SIINFEKL-pulsed DL1-DCs or control-DCs and transduced with vector alone (Mig), Eomes or Eomes-DN. Thin lines, isotype-matched control antibody; Thick lines, anti–granzyme B. Numbers above bracketed lines indicate percent granzyme B–positive cells among total CD8$^+$ cells. Data are representative of four independent experiments.

CTL differentiation *in vitro* and *in vivo*, even if Notch1 is also expressed on CD8$^+$ T cells.

Notch2 promotes the expression of cytotoxic molecules

We next tested if Notch2 signaling directly controls the expression of cytotoxic effector molecules. For this, we transduced the active form of Notch2 (its intracellular domain; N2IC) into a CD8$^+$ T cell–derived hybridoma (B3Z). B3Z cells expressed neither granzyme B nor perforin; however, transduction of N2IC into B3Z cells (B3Z-N2IC cells) induced the expression of both molecules, even in the absence of TCR stimulation (**Fig. 3a,b**). Stimulation of B3Z-N2IC cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin further upregulated granzyme B expression (**Fig. 3a**), whereas this stimulation did not induce granzyme B expression in B3Z cells transduced with control vector (B3Z-mock cells). These results suggest that Notch2 signaling controls the expression of granzyme B mRNA and perforin mRNA.

The T-box transcription factor Eomes controls effector CD8$^+$ T cell function[5]. Although activated CD8$^+$ T cells expressed Eomes, B3Z-N2IC cells did not express Eomes (**Fig. 3b**). As suggested before[5], transduction of Eomes into CD8$^+$ T cells upregulated granzyme B expression, whereas transduction of a dominant negative form of Eomes (Eomes-DN) suppressed granzyme B expression slightly (**Fig. 3c**). However, transduction of Eomes-DN into activated CD8$^+$ T cells from OT-I TCR–transgenic mice did not suppress DL1-mediated upregulation of granzyme B (**Fig. 3c**). Furthermore, overexpression of Eomes in B3Z cells did not induce granzyme B expression, even after stimulation with PMA and ionomycin (**Supplementary Fig. 3a** online). In addition, overexpression of Eomes in B3Z cells upregulated IFN-γ only after stimulation with PMA and ionomycin (**Supplementary Fig. 3a**). The stimulation of CD8$^+$ T cells by DL1-DCs also enhanced IFN-γ expression relative to that induced by control-DCs, but this enhancement was inhibited by transduction with Eomes-DN (**Supplementary Fig. 3b**). These findings collectively demonstrate that Notch2 controls T cell expression of granzyme B independently of Eomes and controls IFN-γ in a way dependent on Eomes.

The proximal region of the *Gzmb* promoter contains several binding elements for the DNA-binding protein RBP-J, which, after binding to the intracellular domain of Notch, is converted from a transcriptional repressor to a transcriptional activator; RBP-J-binding elements are called 'RBEs' (**Supplementary Fig. 4** online). We used a luciferase assay to evaluate whether *Gzmb* promoter activity was influenced by Notch signaling. B3Z-N2IC cells had *Gzmb* promoter activity that was five- to tenfold higher than that of control B3Z-mock cells. Deletion and substitution of RBEs showed that the region containing the most proximal RBE was necessary for N2IC-induced promoter activity (**Fig. 4a,b**). We then confirmed by chromatin immunoprecipitation direct binding of RBP-J to this proximal RBE region in the *Gzmb* promoter in parent B3Z cells (**Fig. 4c**). To confirm direct binding of N2IC to this RBE, we did chromatin immunoprecipitation with anti-Myc in B3Z cells transduced with Myc-tagged N2IC (**Fig. 4d**). We detected the RBE amplicon in the anti-Myc chromatin immunoprecipitates of B3Z cells transduced with Myc-tagged N2IC but not those of B3Z-mock cells. We next determined whether Notch2 controls the transcription of perforin. In a promoter assay using the 2,300–base pair region upstream of the transcription initiation site of the gene encoding perforin, B3Z-N2IC cells had higher activity of the perforin promoter than did control B3Z-mock cells (**Supplementary Fig. 5a** online). We confirmed by chromatin immunoprecipitation that RBP-J directly bound to an RBE 140 base pairs upstream of the transcription initiation site of perforin (**Supplementary Fig. 5b**). These results demonstrate that Notch2 signaling directly regulates transcription of genes encoding the crucial CTL effector molecules granzyme B and perforin.

We found that the region 40 base pairs downstream of the most proximal RBE was also critical for activation of the *Gzmb* promoter in B3Z-N2IC cells (**Fig. 4a**). This region contained a CRE site (**Supplementary Fig. 4**), and mutation of this CRE site also decreased *Gzmb* promoter activity (**Fig. 4e**). The effects of RBE and CRE mutation were similar in magnitude, in terms of *Gzmb* promoter activity, in primary CD8$^+$ T cells from B6 mice stimulated with allogeneic BALB/c BMDCs transduced with DL1 (**Fig. 4f**). These findings indicate that both the RBE and CRE are required for Notch2-mediated expression of granzyme B.

Notch2 interacts with CREB1

We next assessed whether CRE-binding proteins are involved in CTL differentiation. CREB1 activity is regulated by protein kinase A[25]. CREB1 recruits the transcriptional coactivator p300 (A001716) through phosphorylation of the serine residue at position 133 of

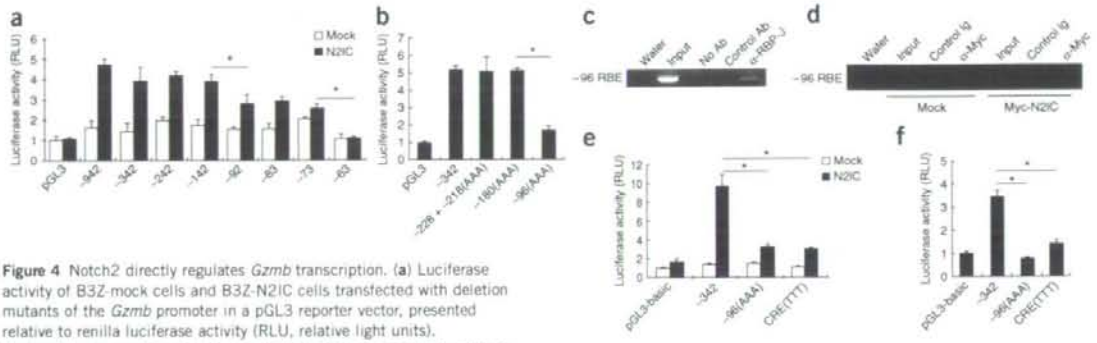

Figure 4 Notch2 directly regulates *Gzmb* transcription. (**a**) Luciferase activity of B3Z-mock cells and B3Z-N2IC cells transfected with deletion mutants of the *Gzmb* promoter in a pGL3 reporter vector, presented relative to renilla luciferase activity (RLU, relative light units). Horizontal axis, position of initial nucleotide of deletion mutant, relative to the transcription start site ('pGL3-basic' indicates vector only). *, $P < 0.05$. (**b**) Luciferase activity of B3Z-mock and B3Z-N2IC cells transfected with pGL3 vector encoding regions of the *Gzmb* promoter with mutations at various RBE sites (**Supplementary Fig. 4**), relative to that of cells transfected with the wild-type *Gzmb* promoter. *, $P < 0.05$. (**c**) Chromatin-immunoprecipitation analysis of fixed and sonicated B3Z cells immunoprecipitated with anti-RBP-J or control antibody (Ab); complexes were treated with protease and the resultant DNA was amplified by primers specific for the *Gzmb* promoter region containing an RBE 96 base pairs upstream of the transcription initiation site (-96 RBE). Water, no template DNA; Input, total DNA before immunoprecipitation. (**d**) Chromatin-immunoprecipitation analysis of B3Z cells transduced with mock or Myc-tagged N2IC vectors, then fixed and lysed; enzymatically sheared chromatin DNA was immunoprecipitated with anti-Myc or control mouse IgG, followed by DNA amplification as described in **c**. (**e**) Luciferase activity of B3Z-mock and B3Z-N2IC cells transfected with pGL3 plasmid vectors containing mutated *Gzmb* promoters (**Supplementary Fig. 4**), presented relative to renilla luciferase activity. CRE(TTT), CRE with three thymine substitutions. *, $P < 0.05$. (**f**) Luciferase activity of $CD8^+$ T cells first purified from BALB/c splenocytes stimulated for 3 d with B6 BMDCs, then, after purification, transfected with *Gzmb* promoter–driven luciferase constructs (**Supplementary Fig. 4**) and cultured for 1 d more, presented relative to the activity of cells transfected with pGL3 vector only. *, $P < 0.05$. Data are representative of five (**a**–**e**) or four (**f**) independent experiments (error bars, s.d., **a**,**b**,**e**,**f**).

CREB1 by cAMP-activated protein kinase A[25]. Transduction of a dominant negative form of CREB1 (A-CREB1), which contains a substitution of alanine for the serine residue at position 133 and is thus incapable of recruiting p300, impaired granzyme B expression in both B3Z-N2IC cells and primary OT-I $CD8^+$ T cells (**Fig. 5a**,**b**). The effect of A-CREB1 was less apparent in OT-I T cells; this blunted effect might be attributed to the delayed overexpression of A-CREB1, as retrovirus-mediated transduction of A-CREB1 in T cells required preactivation of T cells. Nevertheless, these data demonstrate that Notch2-mediated CTL differentiation requires a CREB1-mediated signal.

To examine the molecular mechanism whereby Notch2 and CREB1 signaling integrate during CTL differentiation, we determined if N2IC interacted with CREB1. When we transfected both CREB1 and Myc-tagged N2IC into human embryonic kidney 293T cells, CREB1 immunoprecipitated together with anti-Myc immunoprecipitates (**Fig. 5c**). In contrast, A-CREB1 did not interact with N2IC (**Fig. 5d**), which indicates that phosphorylation of CREB1 is required for the formation of a complex of CREB1 and N2IC.

Next we used gel-shift assays to examine binding of the N2IC-CREB1 complex to the *Gzmb* promoter. We found a dominant complex with a probe containing wild-type RBE and CRE sites, based on the sequence of the proximal *Gzmb* promoter (**Fig. 6a**). We also detected a faint band on the probe containing wild-type RBE and CRE sites as well as on a probe of wild-type RBE and mutant CRE, but not on a probe of mutant RBE and wild-type CRE (**Fig. 6a**). The mobility of this faint band shifted after the addition of anti-RBP-J, even in B3Z cells (data not shown), which indicated that this band

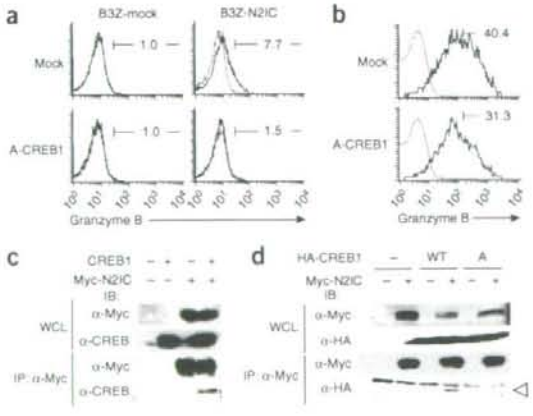

Figure 5 CREB1 is required for Notch2-mediated granzyme B expression through interaction of the intracellular domain of Notch2 with pCREB1. (**a**) Flow cytometry of granzyme B expression by B3Z-N2IC or B3Z-mock cells mock-transfected (top row) or transfected with A-CREB1 in a bicistronic retroviral vector encoding human NGFR, assessed in cells gated as GFP^+ (N2IC) and/or positive for human NGFR (A-CREB1). Thin lines, thick lines, anti–granzyme B. Data are representative of five independent experiments. (**b**) Flow cytometry of granzyme B expression by GFP^+CD8^+ gated cells among OT-I-transgenic T cells stimulated with SIINFEKL peptide and transduced by retroviral vectors encoding GFP with or without A-CREB1. Thin lines, isotype-matched control antibody; thick lines, anti–granzyme B. Data are representative of five independent experiments. (**c**) Immunoblot analysis of N2IC and CREB1 in 293T cells transfected with Myc-tagged N2IC and/or CREB1, detected with anti-Myc and anti-CREB in whole-cell lysates (WCL) and after immunoprecipitation with anti-Myc (IP: α-Myc). Data are representative of five independent experiments. (**d**) Immunoblot analysis of CREB1 and N2IC in 293T cells transfected with hemagglutinin-tagged wild-type CREB1 (WT) or mutant CREB1 (A) with or without Myc-tagged N2IC, detected with anti-Myc and anti-hemagglutinin (α-HA) in whole-cell lysates and after immunoprecipitation with anti-Myc. Arrowhead indicates hemagglutinin-tagged CREB1. Data are representative of three independent experiments.

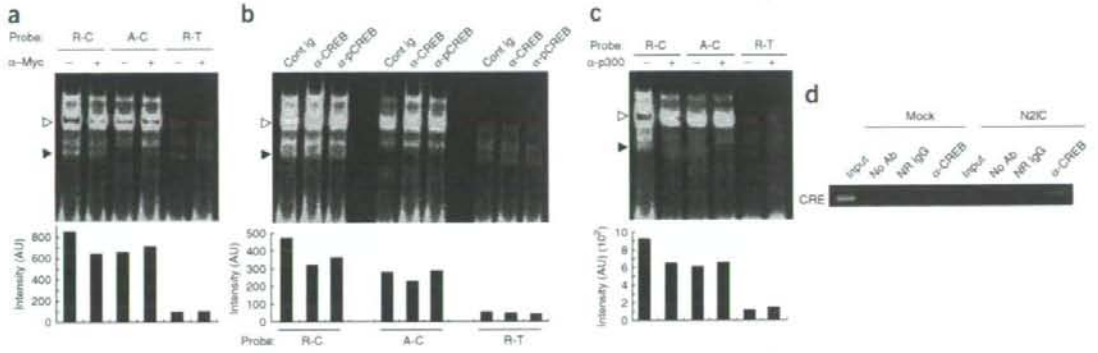

Figure 6 Notch2 interacts with pCREB1 and stably recruits p300 to the *Gzmb* promoter. (**a–c**) Gel-shift assay of nuclear extracts of B3Z-N2IC cells incubated with various probes (top (**a**,**c**) or bottom (**b**)) in the presence or absence of various antibodies (above lanes). R-C, wild-type RBE and wild-type CRE; A-C, mutant RBE and wild-type CRE; R-T, wild-type RBE and mutant CRE. Open arrowheads, dominant CRE-dependent complex; filled arrowheads, RBE-dependent complex. Below, intensity of bands indicated by open arrowheads, presented as arbitrary units (AU). Cont, control. Data are representative of five independent experiments. (**d**) Chromatin-immunoprecipitation analysis of fixed and sonicated B3Z-mock or B3Z-N2IC cells immunoprecipitated with anti-CREB or control antibody; complexes were treated with protease and the resultant DNA was amplified by primers specific for the *Gzmb* promoter region containing a CRE. NR IgG, control isotype antibody. Data are representative of five independent experiments.

corresponded to RBP-J. To determine the composition of the dominant complex, we analyzed the mobility shift or intensity of the band by adding specific antibodies and mutant probes to the nuclear extracts. The addition of antibody to Myc-tagged N2IC decreased the intensity of the dominant complex on the probe containing wild-type RBE and CRE sites to the intensity of the probe of mutant RBE and wild-type CRE (**Fig. 6a**), which indicated that N2IC was one of the components of this complex. Furthermore, the intensity of the dominant band binding to the probe of wild-type RBE and mutant CRE was apparently lower, even if RBP-J bound to this probe (**Fig. 6a**), which indicated that CRE was essential for binding of the dominant complex to DNA. The addition of anti-CREB1 or antibody to CREB1 phosphorylated at the serine residue at position 133 (pCREB1) decreased the intensity of the binding of the dominant complex to that of the probe containing wild-type RBE and CRE sites and, to a lesser extent, to that of the probe of mutant RBE and wild-type CRE (**Fig. 6b**). These findings indicate a requirement for Notch2 signaling for optimal binding of pCREB1 to the probe containing wild-type RBE and CRE sites and also show that pCREB1 is also a component of this dominant complex. The addition of anti-p300 attenuated binding of the dominant complex only to the probe containing wild-type RBE and CRE sites (**Fig. 6c**), which demonstrated that p300 is a component of the dominant complex recruited to the *Gzmb* promoter only when both RBP-J or N2IC and pCREB1 bind simultaneously. As the addition of anti-Notch2 or anti-p300 did not change the intensity of the dominant band on the probe of mutant RBE and wild-type CRE, CRE-binding factors including CREB1 might form a complex without N2IC and p300, and this complex might bind to the probe of mutant RBE and wild-type CRE and migrate to a location similar to that of the dominant complex in the native polyacrylamide gel. These results collectively suggest that the dominant complex identified by the probe containing wild-type RBE and CRE sites is composed of N2IC, pCREB1 and p300. Finally, we tested by chromatin-immunoprecipitation assay whether CREB1 bound directly to the *Gzmb* promoter. We detected binding of CREB1 to the CRE in the *Gzmb* promoter in B3Z-N2IC cells but not in B3Z-mock cells (**Fig. 6d**), which indicates that Notch signaling induces CREB1 to binding to this promoter region.

We propose a model in which in the absence of Notch signaling, RBP-J, together with corepressors, binds to RBEs and suppresses granzyme B transcription (**Supplementary Fig. 6** online). In the presence of Notch2 signaling, both RBEs and CREs are required for stable binding of N2IC, pCREB1 and p300 to the *Gzmb* promoter region; in fact, these three molecules formed a complex on the promoter (**Supplementary Fig. 5**). Single p300 molecules can form a complex with N2IC and pCREB1, as p300 is able to interact with Notch or pCREB1 at distinct domains[26,27]. Moreover, p300 may stabilize the DNA binding of the RBP-J-N2IC-pCREB1 complex and/or strengthen granzyme B transcription. The absence of an RBE probably allows unphosphorylated CREB1 or other CRE-binding factors to bind, which may induce a small amount of *Gzmb* promoter activity, but the presence of RBE and Notch2 signals probably results in exchange of the binding protein from CREB1 to pCREB1, perhaps because a pCREB1-containing complex is more stable on the promoter.

DISCUSSION

The $CD8^+$ T cell response to infection and transformed cells requires the recognition of antigen, massive proliferation and differentiation into CTLs[1]. However, the transcriptional programs that control the differentiation of CTLs during the immune response have not been fully identified. In this report, we have shown that DL1-induced Notch2 signals activated the transcription of granzyme B independently of Eomes. We have also identified previously unknown signaling crosstalk in which Notch2 formed a complex with pCREB1 and p300 on the *Gzmb* promoter; this complex was essential for optimal granzyme B transcription. Our results indicate that Notch signaling is required for the dynamic integration of transcription of genes encoding CTL effector molecules and suggest that Notch2 might allow 'tailoring' of CTL differentiation to appropriate environmental stimuli.

Accumulating data have now provided considerable evidence that the differentiation of mature $CD4^+$ T cells is controlled by Notch signaling[12,14,16,18,28,29]. One study has addressed the functions of Notch signaling in $CD8^+$ T cells by hyperactivating Notch with DL1 (ref. 20). That study found that the ligation of Notch on splenic $CD8^+$ T cells by purified DL1 protein results in less IFN-γ, which suggests an

inhibitory function for Notch signaling in CD8⁺ T cell activation. However, that study did not address whether that inhibitory effect of DL1 is recapitulated by direct interaction of DL1 and Notch on CD8⁺ T cells *in vivo*. Furthermore, purified DL1 protein might not be able to completely mimic the effect of membrane-bound DL1. Here we have presented *in vitro* and *in vivo* evidence indicating that downregulation of DL1 on DCs or Notch2 deficiency on CD8⁺ T cells resulted in much lower expression of molecular effectors necessary for effective CTL function. Our data have provided definitive evidence that Notch2-mediated signaling is important in promoting CTL function. Naive CD8⁺ T cells express Notch2, which is rapidly upregulated after TCR-mediated signaling. The expression of DL1 and DL4 in DCs is also upregulated by Toll-like receptor signaling[14]. Our data suggest that such upregulation of Notch and Notch ligands by environmental stimuli would be an important step in the promotion of CTL activity in situations necessitating the eradiation of intracellular pathogens. In addition, we have shown that activated CD8⁺ T cells had high expression of Notch1, but not of Notch3 and Notch4, which suggests a possible contribution of Notch1 to CTL differentiation.

Eomes is a T-box transcription factor that is highly homologous to T-bet and is expressed in activated CD8⁺ T cells and activated natural killer cells[5,6,30]. Overexpression and antagonism studies with dominant negative proteins suggest that Eomes and T-bet might have cooperative or redundant functions in regulating the genes encoding IFN-γ and cytolytic molecules in CD8⁺ T cells[5]. However, those studies did not show that Eomes or T-bet directly regulates the transcription of granzyme B or perforin by reporter assays, although they demonstrated by chromatin immunoprecipitation assay that Eomes does bind to *Gzmb* promoter regions[6]. We have shown here that Notch2-mediated signaling directly activated the transcription of genes encoding granzyme B and perforin even in the absence of Eomes expression in B3Z cells and in the presence of a dominant negative form of Eomes in mature T cells. Our data indicate that Notch2 signaling is able to 'prime' the transcription of genes encoding cytotoxic molecules in the absence of Eomes. However, the Notch2-induced higher IFN-γ expression in CTLs was inhibited by a dominant negative form of Eomes. These data suggest that Notch and Eomes have both distinct and overlapping functions in exerting full effector functions of CD8⁺ T cells.

The precise regulatory mechanisms for the ontogeny, function and survival of effector and memory CD8⁺ T cells, as well as their lineage relationship, remain controversial[2]. Although we have shown that Notch-mediated signaling directly controlled CTL effector functions, we do not yet know whether Notch signaling is involved in aspects of the memory cell differentiation program. Future efforts should be aimed at determining whether Notch signaling skews naive CD8⁺ T cells toward CTLs instead of memory CD8⁺ T cells or contributes to the differentiation of both cell types.

Several papers have suggested there is crosstalk of Notch signaling with other signaling pathways, including those induced by stimulation with bone morphogenetic protein or transforming growth factor-β. Notch interacts with the intracellular bone morphogenetic protein mediator SMAD1 and the transforming growth factor-β mediator SMAD3. SMAD1 interacts with the Notch intracellular domain to activate transcription of the genes encoding the Hes and Hey basic helix-loop-helix factors[31–33]. Whether DNA binding by SMAD1 is essential for synergy with Notch is controversial[32,34]. Notch signaling also acts together with the transcription factor HIF-1α to mediate cellular responses to hypoxia[35]. Our data have provided evidence of a mechanism of crosstalk between the Notch and CREB1 signaling pathways. Notch2 formed a complex with pCREB1 and p300 on the *Gzmb* promoter. The cooperative nature of this complex was shown by the finding that Notch2 and pCREB1 were able to stably bind to the *Gzmb* promoter only when both proteins were present, and both the RBP-J-binding region and CRE were required for full *Gzmb* transcription. Furthermore, blocking the phosphorylation of CREB1 resulted in lower expression of granzyme B, which indicates the biological importance of CREB1 in CTL differentiation.

CRE- and RBP-J-binding elements are widely distributed in the promoter or enhancer regions of many genes. Therefore, it is possible that the interactions noted in the *Gzmb* promoter region occur in other promoter or enhancer regions and thereby amplify and broaden the repertoire of genes influenced by each pathway. For example, because CREB-responsive elements regulate several cytokines, including IL-2 (ref. 36), that are required for CTL population expansion, the interaction between Notch and CREB-responsive genes might be involved in aspects of CTL function other than cytotoxicity. In addition, Notch signaling is crucial for a variety of cell proliferation, tumorigenesis or cell fate 'decisions'[7,28,37,38], and such events may also require cooperation with the CREB pathway[39,40]. Given the diverse functions of Notch in vertebrate and invertebrate embryogenesis, our data provide a basis for investigating a general function for CREB in Notch-directed cellular programs. Our findings may also advocate the manipulation of CREB pathway components as a means of modulating or redirecting Notch-mediated cellular processes. Finally, our results indicate that the manipulation of Notch signaling might have a considerable effect in clinical settings in which more CTL function is desirable (such as in tumor immunotherapy) or less CTL function is desirable (such as in autoimmune diseases).

METHODS

Mice and cell culture. Female B6 and BALB/c mice 6–8 weeks of age were from Japan SLC. *Notch2*$^{f/f}$ mice have been described[41]. For the generation of E8I-Cre-transgenic mice, the 1.6-kilobase core of E8I enhancer fragment was inserted in front of the *Cd8a* promoter, and cDNA encoding Cre and an internal ribosomal entry site-GFP–poly(A) cassette were inserted after the *Cd8a* promoter to generate the E8I-Cre transgene (I.T., data not shown). The transgene was expressed in CD8α⁺CD8β⁺ αβT cells and CD8αα⁺CD8β⁻ αβ T cells but not in CD4⁺CD8α⁻CD8β⁻ αβT cells (data not shown). OT-I TCR–transgenic mice were from Immuno-Biological Laboratories. All animal work was approved by the Animal Research Committee of The University of Tokushima. DCs were generated from mouse bone marrow. Bone marrow cells were treated with anti-CD4 (GK1.5; 14-0041), anti-CD8 (53-6.7; 14-0081), anti-B220 (RA3-6B2; 14-0452) and anti-CD11b (M1/70; 14-0112; all from eBiosciences), followed by magnetic depletion of labeled cells with anti-rat immunoglobulin G (IgG) Dynabeads (Invitrogen). Isolated cells were cultured for 7 d in the presence of granulocyte-macrophage colony-stimulating factor and IL-4. In some experiments, bone marrow cells were infected three times (days 0, 1 and 2) with retrovirus. At 5 d after the final retrovirus infection, cells were stimulated overnight with anti-CD40 (5 μg/ml; 3/23; 553787; BD Biosciences) and LPS (5 μg/ml). After the stimulation, samples were enriched for CD11c⁺ cells by magnetic separation with CD11c microbeads (MACS; Miltenyi Biotec). In some experiments, GFP⁺ and CD11c⁺ cells were purified with a cell sorter (JSAN; Bay Bioscience). The DCEK fibroblast cell line was infected with the DL1-pKE004–human nerve growth factor receptor (NGFR) bicistronic retrovirus. Cells expressing human NGFR were identified by staining with biotin-conjugated antibody to human NGFR and were enriched with streptavidin-conjugated MACS beads. DL1-transduced DCEK cells were further infected with retrovirus containing DL1-specific shRNA or control shRNA in the vector pFB-SIN. B3Z cells were infected with retrovirus encoding N2IC, Eomes or Eomes-DN and were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS. For depletion of CD4⁺ T cells, splenocytes were labeled with anti-CD4, followed by magnetic depletion of labeled cells with anti-rat IgG Dynabeads (Invitrogen). Samples were enriched for CD8⁺ cells by magnetic separation with CD8 microbeads (MACS; Miltenyi Biotec). For transduction of

genes into OT-I T cells, retroviruses were added 1 d after stimulation. Cells were fixed with paraformaldehyde and were stained with anti–granzyme B or anti-IFN-γ in buffer containing saponin. After washing, expression of granzyme B or IFN-γ was evaluated by flow cytometry.

PCR. Total RNA was extracted with TRIzol (Invitrogen). After reverse transcription with the Omniscript RT Kit (Qiagen), SYBR *Premix Ex Taq* II (Takara Bio) and primer sets (**Supplementary Table 1** online) were used for quantitative PCR. All data are normalized to *Gapdh* mRNA (encoding glyceraldehyde phosphate dehydrogenase) and are presented as 'fold increase' relative to Notch2 expression in naive CD8$^+$ T cells. Pyrobest polymerase (Takara) and primer sets (**Supplementary Table 1**) were used for conventional PCR.

Statistical methods. The distributed data from interval scales were analyzed with Student's *t*-test; a *P* value of less than 0.05 was considered statistically significant. JMP7 software (SAS institute) was used for the log-rank test.

Additional methods. Information on antibodies, vectors, retrovirus preparation, CTL induction *in vitro* and *in vivo*, *T. cruzi* infection, luciferase assay, immunoprecipitation, chromatin immunoprecipitation and gel-shift assay is available in the **Supplementary Methods** online.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001238, A001671, A000690 and A001716.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank T. Kitamura (Tokyo University), S. Reiner (University of Pennsylvania) and I. Fujisawa (Kansai Medical University) for reagents; R. Germain (US National Institutes of Health) for the DCEK cell line and for discussions; K. Ikuta for critical review of the manuscript; C. Kinouchi for technical assistance; and K. Yamakawa for secretarial assistance. Supported by the Japan Society for the Promotion of Science (Grant-in-Aid for Young Scientists (S)), The Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid for Scientific Research on Priority Areas), the Takeda Science Foundation, the Sumitomo Foundation and the Mochida Memorial Foundation.

AUTHOR CONTRIBUTIONS

Y.Ma., Y.Mi., C.I., T.K., A.K. and H.K. did research and analyzed data; H.Y. established antibodies; M.S.-Y., T.S., I.T., and S.C. established genes modified mice; S.S. provided advice for the experiments; and Y.Ma. and K.Y. designed research and wrote the paper.

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